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The Ultrastructural Effects of Cytochalasin B on Fusing Reaggregates of Embryonic Chick Heart and Liver Cells

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THE ULTRASTRUCTURAL EFFECTS OF
CYTOCHALASIN B ON FUSING REAGGREGATES
OF EMBRYONIC CHICK HEART AND LIVER CELLS

A Thesis

Presented to

The Faculty of the Department of Biology
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Masters of Arts

by

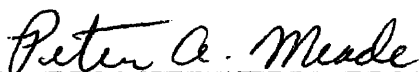
Peter A. Meade

1975

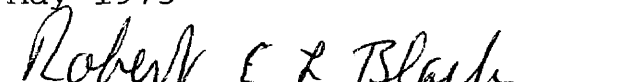
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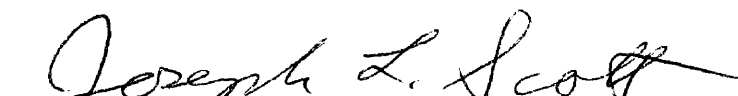
This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Arts


Peter A. Meade

Approved, May 1975


Robert E. Black


Joseph Scott

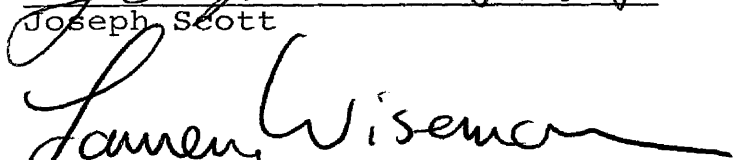

Lawrence L. Wiseman

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	v
LIST OF FIGURES	vi
ABSTRACT	viii
INTRODUCTION	2
MATERIALS AND METHODS	9
RESULTS	16
DISCUSSION	29
LIST OF ABBREVIATIONS	45
BIBLIOGRAPHY	64

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LIST OF TABLES

Table	Page
1. Ability of Fusing Reaggregates to Remain in Contact During Preparation for EM	18
2. Combined Percentage of Heart and Liver Cell Surfaces in Contact with the Surfaces of Other Cells	22
3. Microfilament Presence, Myofilament Structure, and Cytoplasmic Inclusion Size and Presence ..	25

LIST OF FIGURES

Frontispiece - Fusion point of two homogeneous
heart and liver reaggregates.

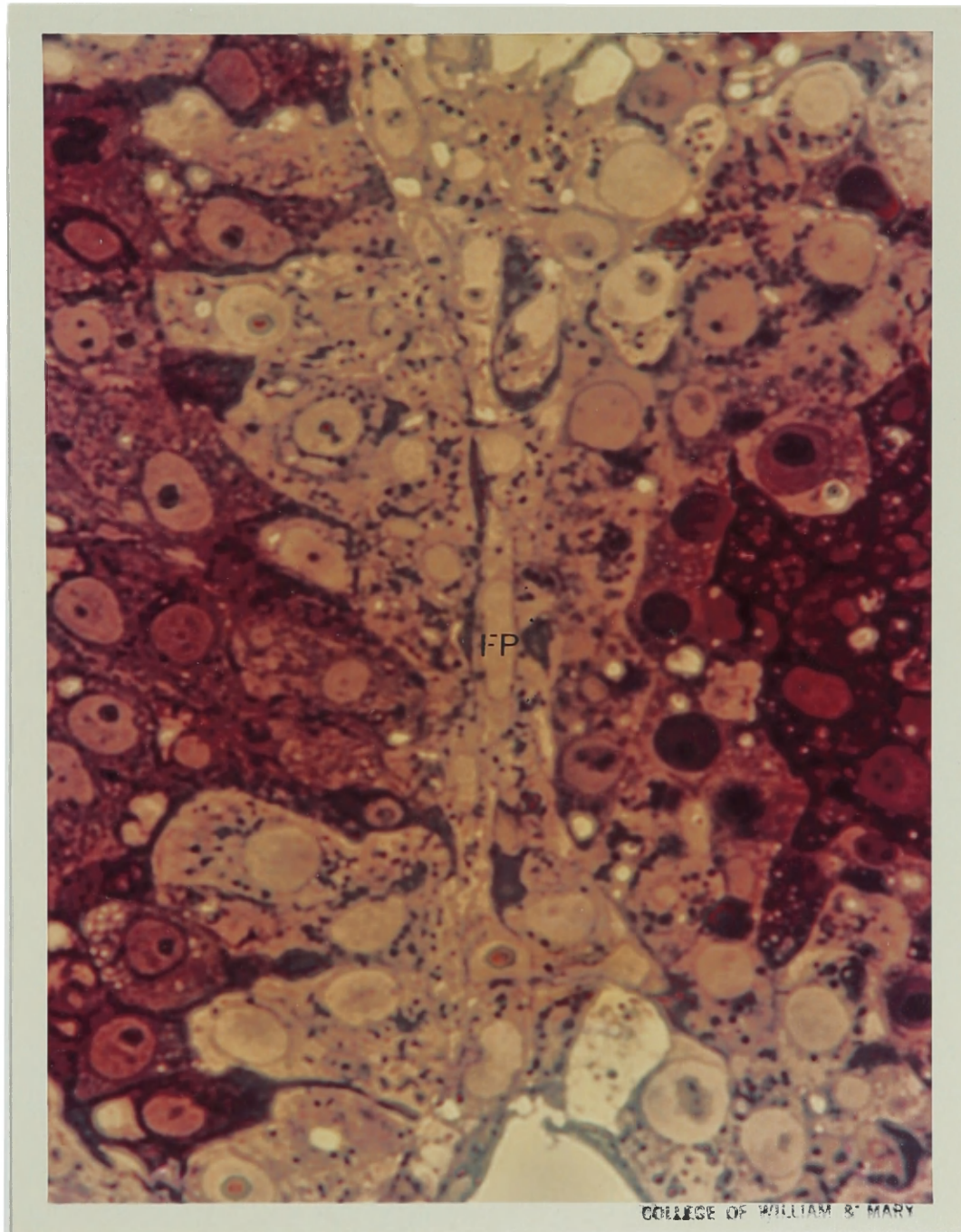
Figure		Page
1	-Obtuse section through a heart and liver fusion mass, indicating regional differences in cell density. (LP = loosely packed; TP = tightly packed).....	47
2	-5 μ g liver reaggregate showing dense cell packing.....	49
3	-5 μ g liver reaggregate showing less dense cell packing.....	49
4	-8 μ g heart tissue mass showing loose density cell packing, and peripheral band of cells.....	51
5	-Control heart tissue mass. Tightly packed cells showing separation of pericardial sheath.....	51
6	-Control liver tissue mass showing microfilamentous alignment.....	51
7	-1 μ g liver recovery showing microfilamentous structure, parallel membrane units and focal point adhesions.....	53
8	-5 μ g liver recovery tissue mass showing microfilamentous structure.....	53
9	-Intact embryonic heart tissue mass showing normal ultrastructural relationships.	55
10	-Control heart tissue mass showing normal ultrastructural relationships for reaggregates.....	55
11	-Enlargement of myofiber of heart reaggregate from control culture.....	55

Figure		Page
12	-1 μ g heart tissue mass showing myofila- ments.....	57
13	-5 μ g heart tissue mass showing myofibril- lar disruption.....	57
14	-5 μ g heart tissue mass showing myofibril- lar orientation, dense intercellular fibers, and x-bodies.....	57
15	-Intact heart reaggregate showing typical <u>maculae adherens</u>	59
16	-8 μ g heart reaggregate showing probable <u>maculae adherens</u> type junction.....	59
17	-1 μ g liver recovery reaggregate showing dense intercellular fibers.....	61
18	-1 μ g liver tissue mass showing dense intercellular fibers, and parallel mem- brane units.....	61
19	-1 μ g heart tissue mass showing both inter- cellular microfibers and collagen fibers.	61
20	-5 μ g liver tissue mass showing dense inter- cellular fibers and distended nuclear envelope.....	63
21	-8 μ g reaggregate showing the poor condi- tion of cells, the x-bodies, and rem- nants of focal point adhesions.....	63

ABSTRACT

Animal morphogenesis relies in great part upon the co-operative phenomena of cell adhesion and cell motility. These components of morphogenesis have been studied in vitro, using chick embryonic tissues and cells, especially in such systems as cell reaggregation and reaggregate fusion. The drug cytochalasin B has been reported to stop motility of cells in these in vitro systems, and with minimum effect on adhesion (Steinberg and Wiseman, 1972). This investigation was undertaken to examine ultrastructurally fusing reaggregates of embryonic cells whose behavior has been predictably modulated by the drug. After treatment with various doses, the following morphological alterations were found within the re-aggregates and their component cells: 1) reduction in the area of cell-to-cell contact; 2) reduction in the variety of membrane junctions formed with focal tight junctions and parallel membrane units predominating; 3) disappearance of 30 to 60 Å microfilaments; 4) the production of two regions within cell masses consisting of loosely and tightly packed cells; and 5) the possible disruption of actin filaments in cardiac cells. Each of these drug-induced structural alterations can be reversed, partially or totally, during recovery in drug-free culture conditions.

THE ULTRASTRUCTURAL EFFECTS OF
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Fusion point of two homogeneous
heart and liver reagggregates. -
Magnification: 750 X

INTRODUCTION

Cell adhesion and cell movement, fundamental processes of vertebrate morphogenesis, are being studied in a number of laboratories using tissue culture model systems. In some investigations embryonic tissues are separated into their component cells, and the behavior of the isolated cells, recombined in various ways, is followed. Suspensions of individual cells derived from the dissociation of tissue fragments have the ability to reaggregate into spheroid tissue masses in culture. If the spheres or cellular reaggregates contain two kinds of cells they will ordinarily sort themselves into distinct groups according to cell type within the mass following aggregation. When two different homogeneous aggregates (i.e. aggregates formed from cells of a single type) are permitted to fuse by placing them in intimate contact, one tissue mass will usually envelop the other, either partially or completely (Steinberg, 1970 and 1974, and Steinberg and Wiseman, 1972).

The behavior of cells during sorting-out and tissue sheet movement has been shown to resemble the behavior of immiscible liquids in an emulsion. When two such immiscible liquids are combined, the liquid with the greater surface tension (i.e. the liquid whose component molecules have a greater attraction for one another) tends to form internal islands, surrounded by the liquid with the lesser surface tension. The final equilibrium state will tend toward that which is most thermodynamically stable (Gordon, et al., 1972, and Steinberg, 1963, 1964, and 1970). Such is also the case when two cell suspensions prepared from two different tissues are mixed, form a heterogeneous reaggregate, and then sort-out. The cells exhibiting the greater cell-to-cell cohesiveness should tend to take the internal position surrounded by those cells with a lesser cohesiveness.

Cytochalasin B is a metabolite of the mold Helminthosporium dematioides (The Cytochalasins, 1971). Although controversial in its mode of action (Burnside and Manasek, 1972, Estensen, et al., 1971, Holtzer and Sanger, 1972, Spooner, 1973, and Wessels, et al., 1971), it has been shown to stop the movement of vertebrate cells in tissue culture (Allison, et al., 1971, Carter, 1967, Gail and Boone, 1971, Estensen,

et al., 1971, Sanger and Holtzer, 1972, Spooner and Wessels, 1970, 1972, Spooner, et al., 1971, Wessels, et al., 1971, and The Cytochalasins, 1971). The ability of the drug to stop cell movement can be used as a tool for examining cell adhesion and cell movement in morphogenesis. Even before the discovery of cytochalasin B (CCB), it was thought possible to separate the processes of cell adhesion and cell movement using in vitro systems. The initial reaggregation of cells, as mentioned above, takes place in a suspension, thus eliminating most substrate based motile activity. Such initial contacts may be purely adhesive in nature. (Unpublished results of Wiseman and Hammond indicate that cells killed by Zenker's fixative can reaggregate to some extent: an indication that cell motility may not be required for initial cell adhesions.)

In order to understand the problems involved in experimentally separating adhesive and motile properties, one must first recognize what is required of cells if they do sort-out. First they must be able to form adhesive contacts with cells of their own kind, as well as with those of another type. This, as we

have seen, is the case when cells reaggregate in suspension culture. Ultrastructural examination of the initial reaggregation of co-mingled cells from two different tissues has shown that cells form initial junctions randomly with the cells of either type (Armstrong, 1970, 1971). These cells must presumably be able to differentiate among the strengths with which they adhere to neighboring cells. Carter (1969) has shown that cells do have the ability to follow artificially produced adhesive gradients in culture, a phenomenon which he calls "haptotaxis". However, in order for this to occur, these cells must of course move. Motility may be classified into two categories: active or passive (Steinberg and Wiseman, 1972). An actively motile cell is one in which the actual mechanisms of movement are internally initiated or mediated (i.e. the energy for movement is supplied by the cell itself). A passively motile cell is one in which movement is directed by external forces (i.e. the energy for movement is supplied from without). Additional work has shown that active cell movement is probably necessary for sorting-out in most cases (Steinberg and Wiseman, 1972, and Maslow and Mayhew,

1972), but probably not all (Armstrong and Parenti, 1973). Although dead cells have been shown to aggregate, we have not observed sorting-out in populations of dead cells. This too might support the hypothesis that energy must be expended for cells to move within a tissue mass.

However, various authors have stated that cells in mutual contact with other cells on all sides, should experience "contact inhibition" of cell movement (Abercrombie, 1961). Contact inhibition of cell movement implies a vectorial inhibition of motility in a direction which would advance a cell over or under the cell to which it has come in contact. This inhibition, as originally described, was exhibited in monolayer cultures, and was believed to occur in reaggregate tissue masses where the cells would be inhibited on all sides (Weston and Abercrombie, 1967). However, Wiseman and Steinberg (1973) were able to show that radioactively labeled individual cells placed on the surface of unlabeled reaggregates were able to move within the mass of solidly packed cells. Other recent work has shown that individual cells are capable of movement with respect to neigh-

boring cells even in confluent monolayers (Garrod and Steinberg, 1973, and Steinberg, 1973).

In order to further examine the properties of movement in sorting-out and tissue envelopment, the drug CCB was employed. Steinberg and Wiseman (1972) and Maslow and Mayhew (1972) found that sorting out and tissue envelopment were inhibited when CCB was added, although Armstrong and Parenti (1972) showed that in certain cases sorting-out does occur in the presence of low levels of CCB. The extent of the reduction of cellular movement was shown to be dependent upon the concentration of CCB used in the experiment. Reaggregation (the adhesion of cell-to-cell, regardless of type) and fusion (the adhesion of tissue mass-to-tissue mass, regardless of type) were not inhibited by the drug. It has been postulated that a combination of passive adhesion and active motile properties determine the direction, speed, and extent of cell locomotion. Movement requiring both an active and a passive component has been termed "cooperative cell locomotion" by Steinberg and Wiseman (1972). This is the kind of movement which may be involved in sorting-out and tissue fusion.

The drug cytochalasin B, as mentioned above, usually prevents sorting-out and the spreading of one tissue upon another, and may therefore, serve as a pharmacological tool for the separation of cell movement and cell adhesion. The purpose of this investigation is to examine cell-to-cell contacts at the ultrastructural level during the drug-induced inhibition of cell movement during the fusion of homogeneous cellular masses in culture, and in addition, to examine any intracellular morphological factors which may affect observed changes in cell behavior.

The action of the drug cytochalasin B on cell aggregation, fusion, and sorting-out has been fairly widely reported (eg. Lieberman, et al., 1973, Overton and Culver, 1973, Appleton and Kemp, 1974, Sachs, et al., 1974, Shaeffer, et al., 1973, Steinberg and Wiseman, 1972, Yamada and Wessels, 1973). However, a methodical investigation of its effects on cell-to-cell interactions at the level of the membrane has not been adequately explored, except perhaps by Overton and co-workers (1973).

MATERIALS AND METHODS

Tissue Culture

Hearts and livers excised from five-day old White Leghorn chick embryos (Pocosham Hatcheries, Richmond, Virginia) were trimmed and washed in a solution of cold Hanks' balanced salt solution. Except for a portion of the intact heart material which was immediately minced and fixed, tissue fragments were placed in a depression slide, washed three times in Calcium-Magnesium-Free (CMF) Hanks' solution, finely minced with microscalpels, and then transferred to 15 ml screwcapped tubes containing 10 ml of 0.1% crude trypsin (Difco 1:250) in CMF Hanks'. After five minutes in a water bath at 37°C, the tubes were transferred to a test tube rotator at 60 rpm, and at an angle of 10° - 20° from the horizontal for another 15 minutes at 37°C.

The cell suspensions were centrifuged in an International table top centrifuge for four minutes at 400 x g. The supernatant liquid was replaced with 3 ml of freshly Millipore-filtered (av. pore size 0.45 μ m) medium (10% horse serum in Eagle's

minimum essential medium (MEM) with Hanks' salts; 100 units of penicillin and 100 μ g of streptomycin per ml.). After a 15 second vortex mix, the tubes were centrifuged at 250 x g for three minutes, the supernatant was removed, and another 3 ml of freshly filtered medium was added. The next 15 second vortex was followed by a centrifugation at 40 x g for one minute, which left the dissociated single cells in suspension, while causing the pelleting of heavier clumps of undissociated cells. The cells in the upper layer of the suspension were transferred to another screwcapped tube and counted with a hemocytometer. The cell counts ranged from 0.6×10^6 to 1.6×10^6 cells per ml of suspension.

Tubes of cells were incubated on a test tube rotator at 60 rpm at 37°C for one hour. They were then centrifuged at 400 x g for four minutes to form pellets. The pellets were then incubated for five hours at 37°C in a water bath after which the pellets were removed, placed in medium (as above, except with Earle's salts) in a depression slide, cut with microscalpels into small squares which would yield upon culture aggregates with a diameter

approximating 0.33 mm. The dimensions of the squares varied with the thickness of the pellets.

No more than ten fragments each were placed in 10 ml culture flasks containing 3 ml of freshly Millipore-filtered medium (Earle's salts). The flasks were placed in a 5% CO₂ atmosphere at 37°C on a water bath shaker at 140 rpm to prevent fusion of aggregates during culture. After overnight incubation, the now spheroid aggregates were removed and washed in the solution which corresponds to the solution in which they would eventually be fused to another aggregate. These solutions were:

- 1) The control - medium with Earle's salts; 2) 1 µg cytochalasin B (CCB) per ml of control medium with 0.1% DMSO; 3) 5 µg CCB per ml medium with 0.5% DMSO; 4) 8 µg CCB per ml medium with 0.8% DMSO; 5) 10 µg CCB per ml medium with 1.0% DMSO; 6) 8% DMSO in medium; and 7) 10% DMSO in medium. These stock solutions were kept frozen in concentrated dilutions, thawed and diluted to proper concentration and kept on ice until actual experiments began. DMSO was used as the initial solvent of CCB (Carter, 1967).

One aggregate each of liver and heart cells

was placed in appropriate hanging drops on the lids of plastic petri dishes, and gently tapped into visual contact. The tissue masses were then incubated in a 5% CO₂ atmosphere at 37°C. In the case of control medium and DMSO in medium, incubation was halted after five hours, since previous work had shown that fusion is well under way at this point (Steinberg and Wiseman, 1972). 8 µg CCB and 10 µg CCB per ml medium solutions were stopped after 24 hours of incubation, since it was also reported that by this time the fusions were able to resist some mechanical agitation (Steinberg and Wiseman, 1972). One half of the fusions formed in 1 µg and 5 µg CCB per ml were also arrested at 24 hours. The others were thoroughly washed twice in fresh control medium, and placed in new hanging drops of control medium for five additional hours to permit an examination of the recovery of these cells from the effects of CCB, as compared to the control fusions also incubated for five hours.

Histology

After the fusion masses were removed from the incubator, they were immediately washed in 0.1 M

phosphate buffer at pH 7.4 to remove the serum from the bathing solution of the fusions. They were then placed in 5 ml fixation vials containing 4% glutaraldehyde in phosphate buffer, and then rotated at 15 rpm at room temperature for one hour. All room temperature steps were done with the vial on a rotator, since it was found that this permitted a more thorough exchange of embedding and fixation chemicals into and out of the cells, without excessive agitation of often very fragile fusions. Three fifteen minute washes followed the primary fixation. The vials were stored 1 - 4 days at 4°C before a one hour room temperature post-fix in 1% OsO₄ in a phosphate buffer. Dehydration took place in acetone. The 70% acetone step was replaced with 2% uranyl acetate in 70% alcohol at 4°C, to permit good staining by the uranyl acetate. The fusions were flat embedded in Epon 812.

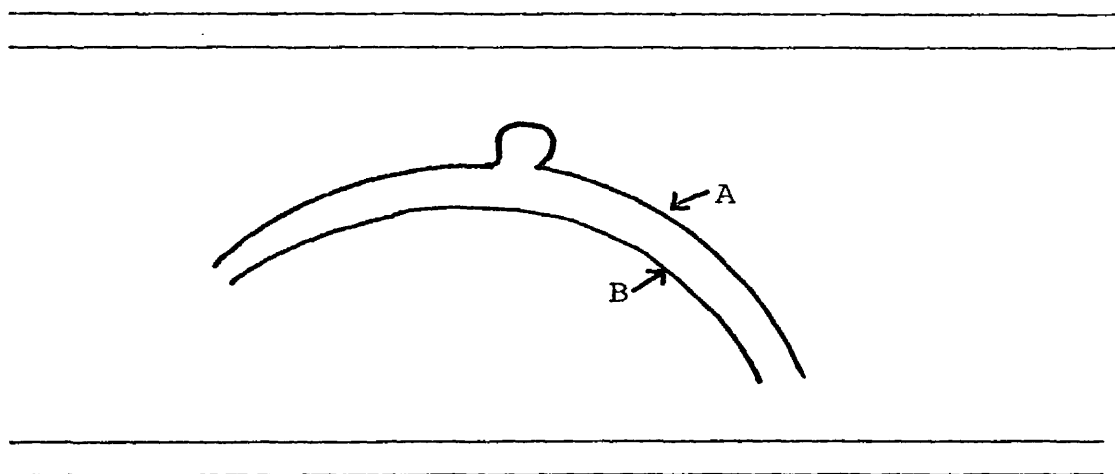
For electron microscopy, 45 - 70 nm sections were cut with a diamond knife on an LKB Ultramicrotome, and then stained in lead citrate or Reynold's lead citrate. Thick sections between 0.5 μ m and 1.0 μ m were cut on the LKB, and stained in toluidine blue for light microscopy.

Cell-to-Cell Measurement

The proportion of cell surface in contact with other cell surfaces was determined by randomly selecting individual cells on a micrograph. The initial cell was chosen at one compass midpoint followed by another (i.e. North, then South, etc.) on the micrograph as it sat, regardless of the orientation of the picture. Each successive cell was chosen so that no point of contact was found between it and any other previously measured cell (two cells sharing the same junctions were not both counted, because an exact doubling of the importance of their mutual adhesions would have resulted). Measurements were taken of 1) the specific length of the cell membrane as it appeared on the micrograph, neither in contact with, nor in adhesive junction with an opposing plasmalemma, and 2) those parts of the cell periphery which were "in contact". Included as points of contact were normal cell junctions (i.e. desmosomes, intercalated discs, gap junctions, and tight junctions, which fit the general descriptions in works by Fawcett [1966], and Friend and Gilula [1972]), and parallel membrane units in which the path followed by one of the plasmalemma was mirrored ex-

actly by its partner on the opposing cell. The separation of these membranes must fall between 100 and 200 Å as this type of junction has been described by various authors: 100 to 200 Å (Armstrong, 1970); 150 Å (Friend and Gilula, 1972); and 150 Å or more by other authors. Any non-mirrored kinks in either membrane were not measured as part of the parallel membrane unit. (Eg. see Diagram 1: If we were examining cell A, we would only measure that part of A which is parallel, or in contact with B.)

DIAGRAM 1
JUXTAPOSED MEMBRANES



RESULTS

The degree of tissue envelopment which occurs within a given period of time when two cellular masses are fused, depends upon CCB dosage (Steinberg and Wiseman, 1972). We have used the tenacity with which the fusing tissue masses remain together as a single fusion mass during fixation and embedding, as a criterion for measuring the degree of CCB effect. We stopped the incubation of fusing, paired, control, and DMSO reaggregates at five hours, because previous work had already demonstrated tissue fusion at this time. In preliminary work, the CCB experiments were stopped after five hours also. However, this proved ineffective, since no fusion masses were recovered intact. This first CCB experiment consisted of 31 pairs of reaggregates in a solution containing 10 μ g CCB per ml of medium with 1% DMSO (henceforth to be noted as 10 μ g). It was found that the reaggregates were not sufficiently fused to withstand a transfer to fixation vials by means of a mouth pipette. In a second experiment at this same dosage, 44 hours of incubation were required to produce four intact fusions of the 36 attempted. However, as shown in

Table 1, no fusion was able to withstand the fixation and embedding procedures.

DMSO and control fusion masses revealed indistinguishable ultrastructural characteristics, although an examination of Table 1 reveals a major difference between control and DMSO fusion strengths. The following discussion will show how this difference in fusion strength can be reconciled. The parenthetically listed data in Table 1 shows the results of preliminary control fusions which survived manipulation as readily as fusions cultured in DMSO. In the first series of experiments, 116 fusions were attempted in medium alone. After the five hour incubation period, 106 (91%) of these had formed intact fusion masses, capable of being transferred intact to fixation vials. After fixation procedures involving agitation only during the transfer to and from the fixation vials, 98% fusions were intact. This data was listed only parenthetically because these nonagitation procedures proved to be inadequate for EM fixation and embedding. Overall results, including those of the preliminary control experimentals did show that fusions incubated in DMSO were only slightly more resistant to separa-

TABLE 1

ABILITY OF FUSING REAGGREGATES TO REMAIN IN CONTACT DURING PREPARATION FOR EM

<u>Procedural Step</u>	<u>DMSO</u>	<u>Control¹</u>	<u>1 μg R²</u>	<u>5 μg R²</u>	<u>1 μg</u>	<u>5 μg</u>	<u>8 μg</u>	<u>10 μg</u>
I. Raw Data								
(Successful Attempts)								
A. Post-incubation	15:15	18:18 (106:116)	14:15	13:15	14:18	9:18	7:18	4:36
B. Post-fixation	14:15	8:18 (98:116)	13:15	13:15	11:18	8:18	5:18	0:36
C. Post-inspection	14:15	8:18 (98:116)	13:15	13:15	11:18	8:13	3:18	-
II. Percentages								
A. Post-incubation	100%	100% (91%)	93.3%	86.7%	77.7%	50.0%	39.9%	11.1%
B. Post-fixation	93.3%	44.4% (91%)	86.7%	86.7%	61.2%	44.5%	27.8%	0.0%
C. Post-inspection	93.3%	44.4% (91%)	86.7%	86.7%	61.2%	44.5%	16.7%	-
III. Incubation Time								
(in hours)	5	5	5(+24) ³	5(24+) ³	24	24	24	44

1. Parenthetical percentage includes data from pilot experiments.
2. R = Recovery Experiments.
3. 5 hours of recovery in control medium, after 24 hours in experimental medium.

tion by agitation than control fusions incubated in medium alone. The comparison of numbers of intact fusion masses formed in medium alone, to those formed in 8% DMSO, as well as a comparison of both these fusions at the ultrastructural level, demonstrated that DMSO seemed to have little or no effect on the cell activities we are interested in.

Of the fusions maintained in CCB, higher doses generally inhibit tissue fusion more effectively than lower doses. The tissue masses fused in CCB (1 and 5 μ g) for 24 hours, then transferred to medium alone for five hours, approached control levels for fusion success (see Table 1).

An examination of high and low magnification micrographs of CCB-treated fusions reveals areas within each mass, characterized by either tightly packed or loosely packed cells (eg. Fig. 1). The cells within the tightly packed regions display all of the cell-to-cell junctions previously mentioned, but those cells in the loosely packed regions, when in contact at all, are joined only by focal point adhesions and broad parallel membrane units. Both heart and liver tissue masses showed similar arrange-

ments. This type of tight and loose association pattern has also been discussed by Overton and Culver (1973). In their report on the effects of CCB on chick embryonic corneal cells, these researchers have shown that CCB can produce loosely packed aggregates when compared to DMSO controls. In addition, their observation showed that after 24 hours, "Cell surfaces become clearly opposed over wide areas." This distinct morphology of the loosely packed cells is shown to be characterized by the predominance of focal point adhesions (small microprotrusions of the cell membrane with a radius of curvature down to $0.1\text{ }\mu\text{m}$ forming points of contact with an opposing cell membrane which may or may not be of similar conformation), and parallel membrane units, as the only two continuously appearing cell contacts (Figs. 3 and 4). The number of focal point adhesions did not appear to be greater in the loosely packed cells than in the tightly packed cells. In fact certain areas showed intercellular fibers in disoriented arrays near microprotrusions. This morphology has been presented as an indication of the disruption of focal point adhesions (Lieberman, et al., 1973). The area of tighter packing contains cells forming

all normal contacts and junctions appearing in the same pattern as in controls (Figs. 2 and 5). The data presented in Table 2 shows a slight, but possibly not significant decrease in the percentage of cell contact in both the tightly and loosely packed areas as dose increases. However, dose dependency was found in the recoverability of the cells to normal densities in tissue masses. This situation might indicate a variation in the energy and/or nutritional requirements for repair. The variation would be dependent upon the initial dose applied to the tissue masses. In addition, the higher dose may do more physiological damage, not easily determined by morphological study.

Because of the logistical problems, it was necessary to assume a direct relationship between the volume of cells at each density, and the number of cells at that density. This volume, therefore, can be converted to a percentage number for purely comparative reasons. The number of cells initially found either tightly or loosely packed, varied with the dose (i.e. the higher the dose, the greater the volume of cells which were loosely packed). This number would therefore have ranged from no specific

TABLE 2

COMBINED PERCENTAGE OF HEART AND LIVER CELL SURFACES IN CONTACT WITH THE SURFACES OF OTHER CELLS (DIVIDED INTO GROUPS BY THE RELATIVE PACKING OF THE CELLS WITHIN THE SPECIFIC REGION)

<u>Regions of Cell Density</u>		<u>Intact</u>	<u>Control</u>	<u>1 μg R¹</u>	<u>5 μg R¹</u>	<u>1 μg</u>	<u>5 μg</u>	<u>8 μg</u>
A.	Loosely Packed	N/A	N/A	37.8%	27.4%	9.0%	8.5%	8.6%
B.	Tightly Packed	41.7%	70.2%	50.0%	29.7%	22.3%	22.0%	N/A
C.	Possible Pericardial	N/A	34.9%	N/A ²	23.0%	N/A ²	N/A ²	15.7%
Raw Data in μ m in Contact/ μ m total cell surface								
A.	Loosely Packed	N/A	N/A	156/413	76/278	47/454	76/896	12/140
B.	Tightly Packed	586/1502	1213/1731	203/406	55/185	55/202	31/138	N/A
C.	Possible Pericardial	N/A	543/1551	N/A	40/173	N/A	N/A	500/3438

1. R = Recovery Experiments.

2. Pericardial-like rings were not distinguished in sufficient quantity to be reported here.

N/A = Not Applicable.

loose region in the control fusions, to no specific tight region in the 8 μ g reaggregates. However, it was observed that those 8 μ g tissue masses, which were believed to be cardiac in origin (some evidence of myosin filaments or myofibers were observed), did exhibit a region of tight contact at the edge of the tissue mass (Fig. 5 shows this region in a control tissue mass; Fig. 4, on an 8 μ g mass). Because this tightly packed region is represented by a thin outer stratum only in heart tissues, it therefore suggests a layer of pericardial cells. Steinberg (1970) reported that some cardiac reaggregates are surrounded by a layer of cells believed to be derived from the pericardial layer of the heart. This layer forms a shell-like ring around the tissue mass.

One of the major effects of CCB was the disruption of microfilamentous structure. It has been said, "One man's microfilaments might not be another's" (Holtzer and Sanger, 1972), and in order to avoid confusion, we have classified all filaments within the range of 30 - 60 $\overset{\circ}{\text{\AA}}$ as microfilaments. We have excepted only those which are associated with myosin filaments in the cardiac myofibrils (i.e. the 60 $\overset{\circ}{\text{\AA}}$ in diameter actin filaments).

As noted in Table 3, microfilaments are found in control (Fig. 6), 1 μ g recovery (Fig. 7), and 5 μ g recovery (Fig. 8). When examining certain regions in the 1 μ g fusion masses, microfilament presence was presumed positive, however there was not sufficient evidence to state conclusively that the structures observed actually were microfilaments. Such a marginal situation with 1 μ g doses was also reported by Auersburg (1972). No microfilamentous structures were revealed in either 5 μ g or 8 μ g fusion masses.

The morphology of cardiac tissue involves both intra- and inter-cellular characteristics. It has been shown that CCB does not affect the actual process of muscle contraction (Sanger, et al., 1971, and Sanger and Holtzer, 1972). However, more recent work has revealed that there are certain variables which do change when cardiac tissue is exposed to CCB, (Lieberman, et al., 1973). Some of these include changes in the efficiency of electrophysiological actions, as well as a disaggregation of fibers by broken cell junctions and often disrupted myofibrils. These morphological effects were confirmed by this research. Increased dosages of CCB produced a decrease in the orientation of both muscle cell fibers

TABLE 3

MICROFILAMENT PRESENCE, MYOFILAMENT STRUCTURE, AND CYTOPLASMIC INCLUSION SIZE AND PRESENCE

Character	Intact	Control	1 μ g R ¹	5 μ g R ¹	1 μ g	5 μ g	8 μ g
<u>Microfilament presence</u>	-	+	+	+	0 ²	-	-
<u>Myofilament</u>							
A. Alignment ³							
1. Intracellular	*****	****	***	***	**	*	0 ²
2. Intercellular	*****	***	**	**	*	*	-
B. Z-band width ⁴	10.5nm ± 0.03	46nm ± 6	30-50nm ± 15	60nm ± 6	78nm ± 5	82nm ± 6	-
C. Distance between myosin fibers ⁴	26nm ± 3	25nm ± 3	27nm ± 2	25.5nm ± 3	22nm ± 3	25nm ± 2	-
<u>Cytoplasmic Inclusion</u>	-	-	79nm ± 14	155nm ± 35	237nm ± 42	480nm ± 60	691nm ± 103

1. R = Recovery Experiments.

2. Inconclusive result.

3. Graded alignment (Five * = normal; zero * = no alignment).

4. From samples of 30-50 Z-bands or myosin filament pairs.

+ = Presence confirmed.

- = Presence not established.

 \pm = Standard error of the mean.

and fibrils (Table 3, and Fig. 9 for intact; Figs. 10 and 11 for control; Fig. 12 for 1 μ g; Fig. 13 for 5 μ g; and Fig. 14 for 5 μ g recovery).

The structural arrangement of the individual filaments (i.e. actin and myosin) within the myofibril is rigidly ordered. One of the characteristics of this organization is the Z-band which shows an increase in width that is not characteristic of normal fibrils. This variation in Z-band width was also dose-dependent. Measurements were taken of the distance between myosin filaments, to see if any further major change had occurred in the fibrillar structure. No distinct pattern was found in the slight variations of width recorded in Table 2. The intercellular connections, or intercalated discs, which align the muscle fibers between cardiac cells, are disrupted by high doses of CCB. We observed certain intercellular configurations, which were presumed to be either remnants of intercalated discs, or desmosomes. The specific orientation of the layers of disoriented fibrillar material shown in Fig. 16, appears to be a maculae adherens. A low power view of a normal desmosome can be seen in Fig. 15.

In addition to the desmosomes, other elements of cell-to-cell contact seem to be affected by CCB.

These effects are manifested by an apparent constant increase in the proportion of focal point adhesions upon administration of CCB within the range studied, as well as a similar increase in the presence of more parallel membrane units with fewer undulations and non-parallel kinks. Lieberman, et al. (1973) discussed the relationship of dense intercellular material found between cells affected by CCB to microprotrusions having the appearance of focal point adhesions (Figs. 17, 18, 19, 20). Internal membranous organelles appear to swell with an increase in CCB concentration (a characteristic often displayed by dying cells). For example, mitochondria become distended (Figs. 2, 5, 17, 18, 20, and 21). In certain cases the nuclear envelope appears to show an increased separation between its double membrane components (Fig. 20) as compared to the normal spacing found in control nuclei (Fig. 5). In addition, any collagen fibers observed appear unaffected by CCB at 1 μ g (Fig. 19), and at 5 μ g (Fig. 2), as compared to the controls (Fig. 5), and to those noted in micrographs presented by Newsome and Kenyon (1973). These fibers were 200 to 300 \AA in diameter, having repeating striations with a periodicity of 570 to 630 \AA .

The most curious observation was the presence of spheroid and ovoid electron opaque cytoplasmic inclusions. The structures ranged in size from 46 to 92.5 nm in 1 μ g cells, and from 90 to 1197 nm in 8 μ g cells. Even under high magnification it was difficult to determine if the body was membrane bound. This could be due to the fact that they were found only in cells definitely affected by CCB. They did not disappear entirely from recovery cells, although there was a decrease in their average size over the experimentals of respective dosages. (See Figs. 4 and 21 for 8 μ g; Fig. 2 for 5 μ g; and Fig. 14 for 5 μ g recovery.) These bodies were not observed in either control or DMSO fusion masses, even within apparently dying cells exhibiting lysing of all cell membranes and the formation of myelin-like membrane patterns within the cell itself, typical ultrastructural characters of a dying cell.

DISCUSSION

Because cytochalasin B is dissolved in DMSO it is necessary to distinguish between the effects of CCB and the possible effects of DMSO on cell behavior and morphology. Our ultrastructural examination revealed no effects on cells treated with DMSO, confirming the work of Moskalewski, et al., (1972). It has been previously shown that DMSO has no other basic cellular or behavioral effects when used in the concentrations employed in CCB experiments (Auersburg, 1972, Carter, 1967, Gail and Boone, 1971, Lieberman, et al., 1973, Mizel, 1973, Meade, 1973, Steinberg and Wiseman, 1972). Recently, Dye (1973) has shown that 1% DMSO will inhibit the outgrowth of mouse kidney cells in vitro during the first 48 hours of culture. However, by 72 hours DMSO inhibited outgrowth was found to return to normal levels. Also, Shields and Pollock (1974) showed that DMSO slightly decreased the adhesiveness of BHK and PyBHK cells to plastic, although CCB slightly increased cell adhesion.

The first indication of any effect of cytochalasin B on fusing tissue masses was the decreased ability of the masses to remain in contact during

fixation procedures (Table 1). We cannot presume that our indicator (strength of fusion) is a measure of absolute cell cohesiveness. It is more likely that at higher doses of CCB, less surface area is involved in the contact zone between the two tissue masses and because the total strength of adhesion between two masses is dependent upon the strength per unit area and the total area involved in contact, a smaller zone of contact would be more easily disrupted during experimental handling. At any rate, the properties of fusion masses which could withstand experimental manipulation was a good indication of the concentration of CCB used.

The primary site of CCB control is debatable. In 1973, Spooner postulated the primary site to be the plasmalemma because the drug has been shown to penetrate into the hydrophobic regions of the membrane. Once there, it may cause alterations in permeability and also in the attachment of intracellular structures like microfilaments. If the plasmalemma is considered to be the site of the primary action of CCB, many of the other non-membrane effects attributed to the drug, would become secondary effects. Thus CCB could also have a similar effect on the membranes

of cellular organelles, as well as the plasma membrane itself, especially when the vehicle of administration is a substance like DMSO which is known for its ability to penetrate such permeability barriers as cell membranes.

The initial structural variations noticed after CCB treatment were a reduction in the cells' surface in contact with other cells (Table 2). In addition, the kinds of junctions formed were ultimately reduced to two, although the desmosomes formed a third but questionable class. In recent work by Overton and Culver (1973) the effect of CCB on desmosome formation in aggregates of chick embryonic corneal cells was discussed. Desmosomes were found to form after 24 hours of culture in CCB. We have observed the elimination of distinct tight junctions (contiguous cell membranes with the intercellular space, if any, sufficiently narrow to prevent the passage of such molecules as Lanathanum), gap junctions (20 to 40 Å intercellular gaps according to Friend and Gilula, 1972), and other areas of membrane contact, leaving only the focal point adhesions and the broad parallel membrane units.

The focal point adhesion has been shown to be one of the most tenacious junctions between cells. In experiments in which the cells have been physically separated, focal point adhesions proved to be highly resistant to tearing. These adhesions are among the first junctions to form between re-aggregating cells (Armstrong, 1971), and are believed to be transient in nature, forming between migrating cells regardless of type (Armstrong, 1972). These junctions are thought to be effective in initiating adhesions, because of their small radii of curvature, enabling opposing membranes to overcome intrinsic forces of repulsion (Lesseps, 1963). The mechanism which breaks down these contacts as the cell moves on is not known. However, various workers have shown evidence for the roles of the following factors in cell movement: membrane morphology-focal point adhesions of Abercrombie (1969) and Ambrose (1969); intercellular fibers- examined by Lieberman, et al., (1973), and Overton (1969); and intracellular fibers with their contraction and relaxation dependent upon ATP and relaxation grana (Hoffman-Berling, 1963).

The second junction found to withstand treatment with CCB is the parallel membrane unit which

appears to increase its area of contact between neighboring cells following drug treatment. It is also known that following cessation of movement, cells cultured as reaggregates eventually form broad areas of contact with one another (Zachei and Caravita, 1972). This was confirmed in our own unpublished data, in which such junctional configurations were exhibited by liver cells incubated in reaggregates only two and one half days. This evidence suggests that long stretches of parallel membrane contacts are spontaneously formed when closely packed cells remain relatively immobile, and further that cells will maintain and form specialized junctions even in the non-motile state. This postulate appears to be confirmed in the work of Overton and Culver (1973). Therefore the parallel membrane unit may not depend upon active cell movements, but rather the "passive zipping" together of opposed cell surfaces (Steinberg and Wiseman, 1973).

In the regions of loosely packed cells, few, if any intercellular fibers are found between the membranes forming parallel units. This may imply either an immaturity of the parallel membrane units

(if we define immaturity as being measured by the amount and alignment of intercellular fibers), or it may imply a separate and unique junction with a similar morphology. Since there is a concentration of this fibrillar material next to microprotrusions, we might assume that they could be remnants of focal point adhesions. This has already been suggested by Lieberman (1973) for other tissue types. These focal point adhesions are believed the first to be formed, and the last to pull apart. An alternative explanation for fiber localization and presence might be found in the relationship between the activity of filament production, and the activity of the plasmalemma. Such a relationship has been brought forward by Overton and Culver (1973), suggesting that a decrease in activity within the plasmalemma would correspond to a decrease in the number of intercellular fibers.

The effect of cytochalasin B on the cell surface may reflect mechanisms required for the maintenance and/or formation of certain energy requiring cell junctions. Some other effects of CCB have been outlined on the biochemical level, although the actual modes of action as yet have not been fully detailed.

Three reports show that CCB 1) inhibits ^3H -glucosamine incorporation into surface components (Lee and Roberts, 1973), 2) decreases the transport of 2-deoxyglucose (this decrease is dose-dependent) (Mizel, 1973), and 3) reduces up to 50% the incorporation of ^{14}C -glucosamine into the total mucopolysaccharide and glycoprotein constituency of the cell (Sanger and Holtzer, 1972). If there is such a vast effect on sugar uptake, most of the effects of CCB could be related to a deficiency of this energy source. However, Taylor and Wessels (1973) showed that in glucose free medium, salivary gland morphogenesis occurred without the effects seen during CCB treatment; also, Yamada and Wessels (1973) showed that CCB inhibited 96 - 98% of the deoxyglucose incorporated into the glial and nerve cells, although glucose-free medium caused none of the effects typical of CCB.

Other effects on the cell membrane and its function have been related to CCB: reduction of ^3H -thymidine incorporation in cultures of diploid fibroblasts (Hoehn, 1973); decreased numbers of sodium carrying channels per unit area of the cardiac cell membrane (Lieberman, et al., 1973); inhibition of cytokinesis (Hoehn, 1973, Hammer, et al., 1971, and

Krishan and Ray-Chanduri, 1969); inhibition of the action of insulin prior to modulation of cyclic AMP levels (Baldon and Perdue, 1973); and inhibition of photopolarization and germination of Belvita eggs (Nelson and Jaffe, 1973).

Cytochalasin B has also been related to the formation of crystalloids in drug-treated cells (Moskalewski, et al, 1972). Although the morphological relationship between these crystalloids and our dark staining bodies is minimal, the report does suggest the possibility that the dark staining bodies we have found may be some form of intracellular precipitate resulting from drug treatment. Our evidence has shown that these electron opaque bodies are found in numbers and of sizes dependent upon the dose of CCB (Table 3). In addition, recovery experiments revealed a reduction in the average size of these particles. Their exact significance at this point is not known. But the relationship between their existence and CCB treatment is definitive.

After examining the cell surface, we began to look into the cells themselves. To examine the more exact morphological effects on the internal structure of the cell, we must begin again with a surface struc-

ture. The intercalated disc is an important junction between cardiac cells, which serves partly as a connection for the contracting myofibrils. In a series of investigations by Manasek and coworkers (Manasek, 1971, Manasek, et al., 1972, and Manasek and Monroe, 1972), it was found that during the development of cardiac tissue in the chicken embryo, cell shape alteration as well as changes in cell-to-cell orientation were predominant factors. This implies an instability of the cell junctions found in early embryonic cardiac tissue. In these experiments, although the heart tissue was cultured in a medium inhibitory to beating function, it was not prevented from developing morphologically. Zacchei and Caravita (1972) have shown that reaggregating cardiac myoblasts never show complete realignment of intercalated discs, even though the cells proceed along a somewhat normal developmental path during longterm culture. In the present report we have shown that this junction is totally disrupted only under high doses of CCB. In both long and short term culture, myoblast cells will continue to beat after treatment with CCB at medium to low doses. Considering the work of Lieberman, et al., (1973) we

can assume that the beat is possible in the presence of CCB, but that it decreases in efficiency with each increased dose. Thus the disruption of intercalated discs might be associated with the dose dependent effects on the intracellular myofibers which attach to it.

The intercalated disc is structurally perpendicular to the myofibrils. The distance between myosin fibers was basically constant as the dose varied (Table 3). However, since the fibers are perpendicular to the discs, it appears that any stress caused by even a great separation of the fibers would have a minimum effect on the junctions. We did find evidence that there might be some effect on the myofibrils, causing a longitudinal stress on the fibril, and thereby indirectly pulling the disc junctions. This effect was reported as a measurement of the width of the Z-band. Table 3 reveals that the Z-band becomes less distinct with increases in CCB doses. Similar data on the effect of CCB in causing non-alignment of Z-bodies was reported by Sachs, et al., (1974). This disorientation of the Z-band might indicate the breakdown of the actin filaments, which are the major fibrillar components of these bands.

Others, such as Z-filaments have been postulated to be the connective units between the alternating thin filaments of neighboring sarcomeres. The Z-filaments have been thought to be derived from parent fibers. One such postulate states that these are extensions of molecular actin from the end of specific thin filaments (Franzini-Armstrong, 1973). The breakdown of the thin filaments or their anchoring connections by CCB treatment could explain disorientation of the fibers. Sachs, et al., (1974) point out that the non-aligned Z-bodies contain only residues or short lengths of actin. This disruption of the myofibrils at the Z-band is believed by Sachs, et al., (1974) to be sufficient to cause the observed inhibition of contractility. Whatever the cause, the ultimate effect of disruption of the Z-band could easily be a stress on the intercalated disc.

We have noted that actin filaments have an average diameter within the same range as microfilaments (30 to 60 Å). It therefore appears that some of the behavioral alterations involving subtle decreases in muscle contraction can be associated with the same effects that disrupt general microfilamentous organization. These effects of CCB have been the most hotly contested research areas since the discovery of CCB

itself as a research tool. There has also been a continuing battle as to whether CCB actually affects microfilaments (Burnside and Manasek, 1972, Estensen, et al., 1971, Holtzer and Sanger, 1972, Wessels, et al., 1971). But a more fundamental question yet to be asked is: What are microfilaments? Microfilaments have been described with diameters from 30 to 60 Å, and with lengths depending upon the state of polymerization. The roles of these filaments have been equally disputed, and are sometimes described with supportive functions, and other times with no apparent function at all (Novikopf and Holtzman, 1970, Clony, 1965, Schroeder, 1969, Spooner, et al., 1971, Spooner and Wessels, 1972, Wessels, et al., 1971, Griffin, 1972, Luduena and Wessels, 1973, Meade, 1973, and Yamada and Wessels, 1973). In our findings, CCB has been shown to affect all filaments within the range of 30 to 60 Å, including the actin filaments within the Z-band region of the muscle fiber. The actin filaments are intimately bound to the myosin filaments. It is possible that this intimacy is the reason why actin fibers retain their basic integrity in the rest of the myofiber, at doses which so readily affect "free" filaments (those not complexed with other macromolecular or structural components). These myosin fila-

ments are too large to be classified as microfilaments. A further argument for the protective role of the myosin filament is the fact that the Z-band is a structural element dealing only with thin filaments and those filaments derived from the thin filaments. Furthermore, this is the only region which shows variation with dose. Again, the distance between parallel myosin filaments within the same fiber did not vary in any way related directly to CCB treatment. Sachs, et al., (1974) points out that the hexagonal array of actin and myosin filaments in a myofibril bundle is not disrupted by CCB, up to 10 $\mu\text{g/ml}$, even when Z-bodies loose their alignment. We have also seen that 200 to 300 \AA diameter collagen fibers are also unaffected by CCB.

It is interesting to note one phenomenon which at this time is inexplicable. Regardless of whether a reaggregate is treated with 1 μg , 5 μg , or 8 μg of CCB, the lowest percentage of cell-to-cell contact was 8.5%, not varying significantly from dose to dose. In the more densely packed regions of the re-aggregates, the percentage of cell contact was again constant from dose to dose (Table 3). The major difference must therefore be the relative quantities of cells within each region. General observations

showed this to be the case. Dose dependency was also shown in the recoverability experiments. We found that the more loosely packed regions of the experimentals (1 μ g and 5 μ g) showed marked differences in percentages of cell-to-cell contact during these recovery experiments. This same kind of result held true for the tightly packed regions (Table 2). The quantity of cells affected at each level is believed to vary with dose. The energy requirements of repair would be greater for a large number of highly affected cells than for a smaller number of highly affected cells, as would be the case of 5 μ g cells recovering when compared to those in a 1 μ g reaggregate. This energy factor could be significant within a time space of five hours, the time in which the fusion masses were incubated in recovery medium.

We have shown that the primary ultrastructural effects of cytochalasin B treatment can be directly correlated to the cessation of tissue sheet movement, and the disruption of microfilamentous structure. The number of cells affected by CCB and the extent of the effect appear to be the determining factors for the inhibition of active tissue sheet movement. Both the number of cells affected and the inhibition

of tissue mobility have been shown to be dose dependent. We cannot conclusively state that the disruption of microfilaments is the determining factor in causing the individual cells to become non-motile. However, much of the evidence gathered to date points to a relationship between disruption of microfilaments and the cessation of cell movement. For example, as the drug treated tissue masses recovered their ability for envelopment during culture in drug-free media, microfilaments reappeared. In addition, Table 3 shows that intact heart tissue, without active independent cell migration, does not exhibit microfilamentous structures. It was also noted that microfilaments were disrupted in cells which showed few, if any, morphological changes, as well as in the more highly affected, loosely packed cells. The only basic morphological phenomena which appeared to be disturbed in the tightly packed regions of reaggregates were microfilaments. This in itself shows that these structures have a high sensitivity to the drug. The numerous other morphological and physiological phenomena certainly have some effect on the overall metabolic health of the cell. Nevertheless, the majority of these effects may be

secondary in nature. This is not to say that the putative secondary effects are the direct result of the disruption of motile function, but instead that they may be a consequence of the same initial alteration in the cell, which could indirectly cause the cessation of cell movement.

LIST OF ABBREVIATIONS

AF	- Actin Filament
CI	- Cytoplasmic Inclusion
D	- Desmosome
FPA	- Focal Point Adhesion
GA	- Golgi Apparatus
ID	- Intercalated Disc
IF	- Intercellular Filaments
M	- Mitochondrion
MF	- Microfilament
MT	- Microtubule
My F	- Myosin Filament
NE	- Nuclear Envelope
P	- Plasma Membrane
PL	- Pericardial Layer
PMU	- Parallel Membrane Unit
TJ	- Tight Junction
Z	- Z-band

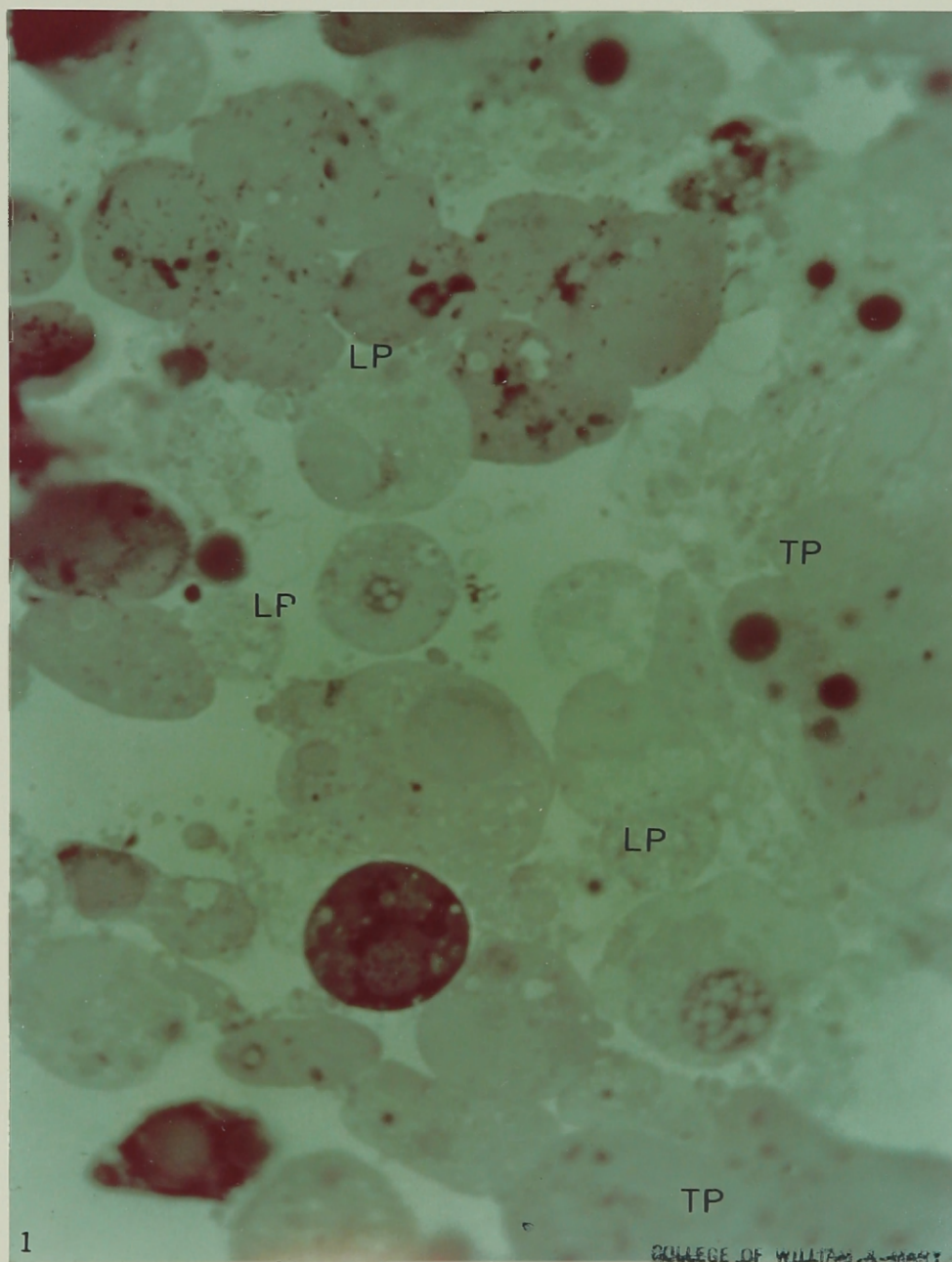


Figure 1 - Obtuse section through a heart and liver fusion mass, indicating regional differences in cell density. (Mag. 750 X).
(LP = loosely packed; TP = tightly packed).

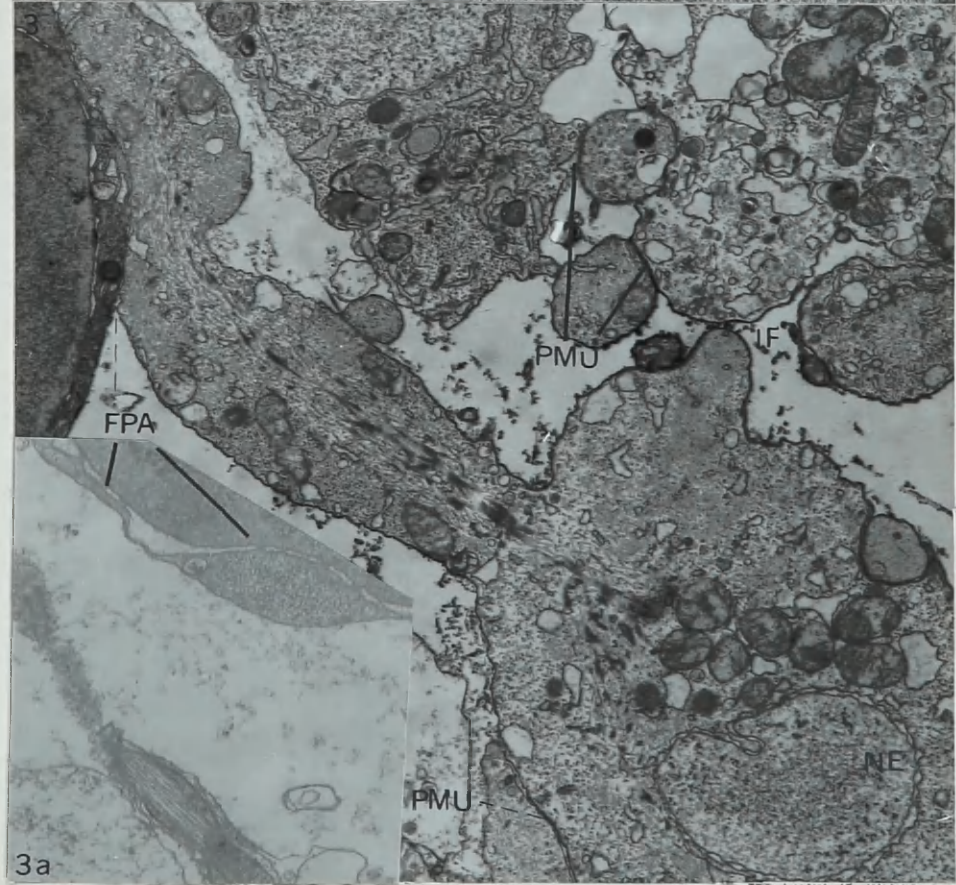
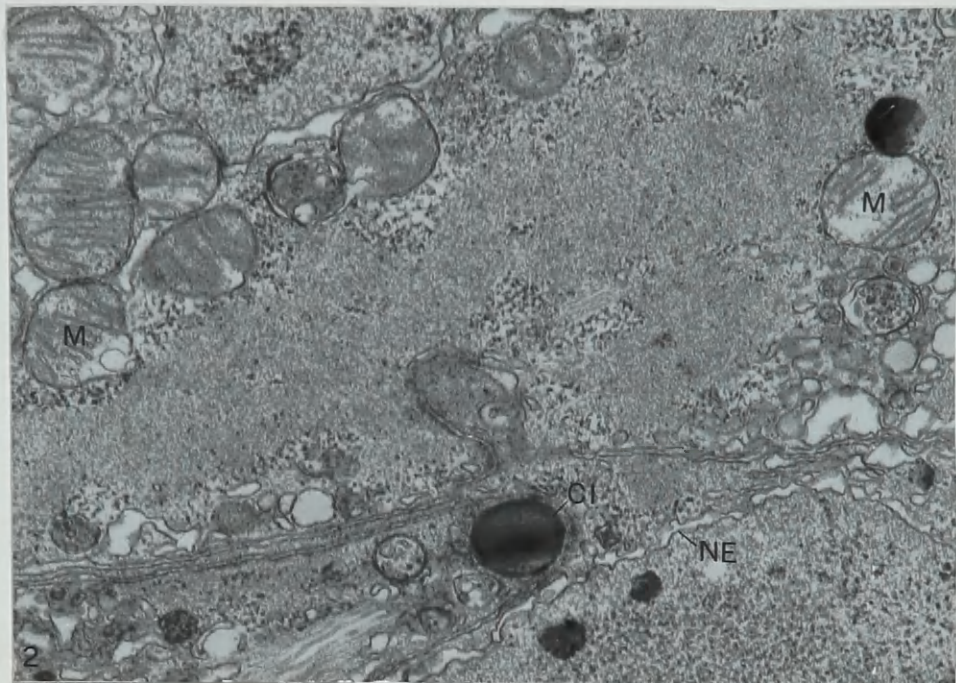


Figure 2 - 5 μ g liver reaggregate showing dense
cell packing. (Mag. 19,000 X).

Figure 3 - 5 μ g liver reaggregate showing less
dense cell packing. (Mag. 11,500 X).

Figure 4 - 8 μ g tissue mass showing loose density cell packing, and peripheral band of cells. (Mag. 4,700 X).

Figure 5 - Control heart tissue mass. Tightly packed cells showing separation of pericardial sheath. (Mag. 9,200 X).

Figure 6 - Control liver tissue mass showing micro-filamentous alignment. (Mag. 63,000 X).

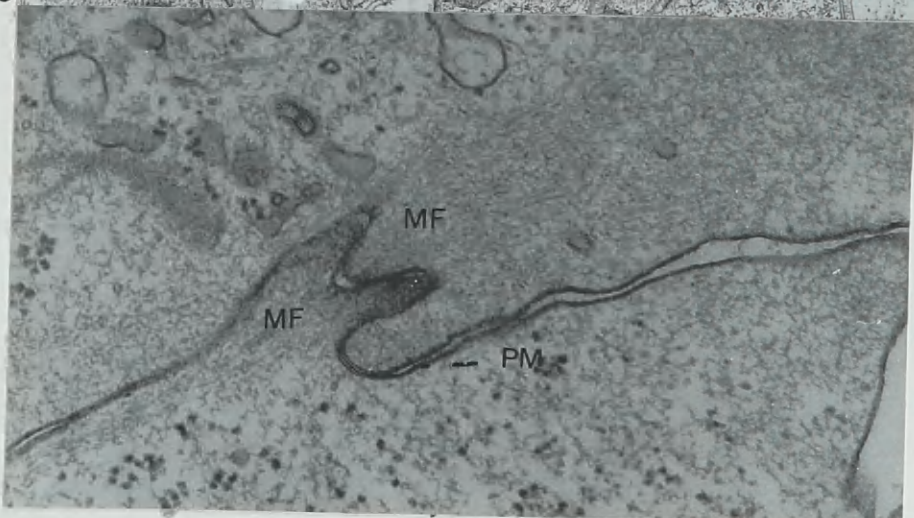
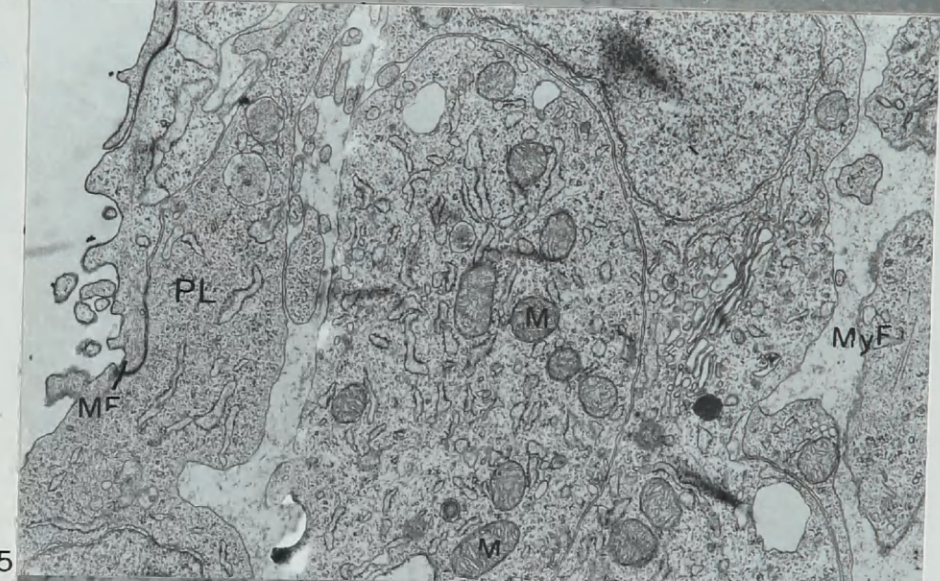
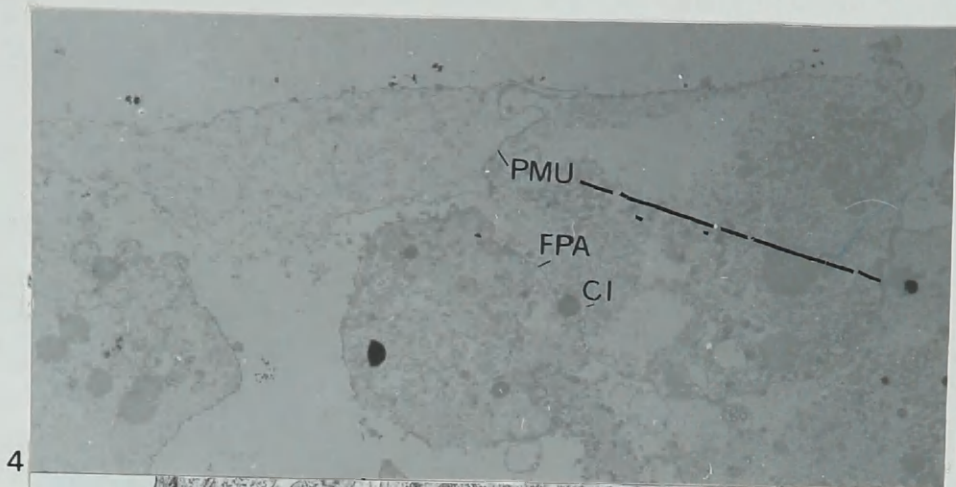


Figure 7 - 1 μ g liver recovery showing microfilamentous structure, parallel membrane units and focal point adhesions. (Mag. 23,800 X).

Figure 8 - 5 μ g liver recovery tissue mass showing microfilamentous structure. (Mag. 68,400 X).

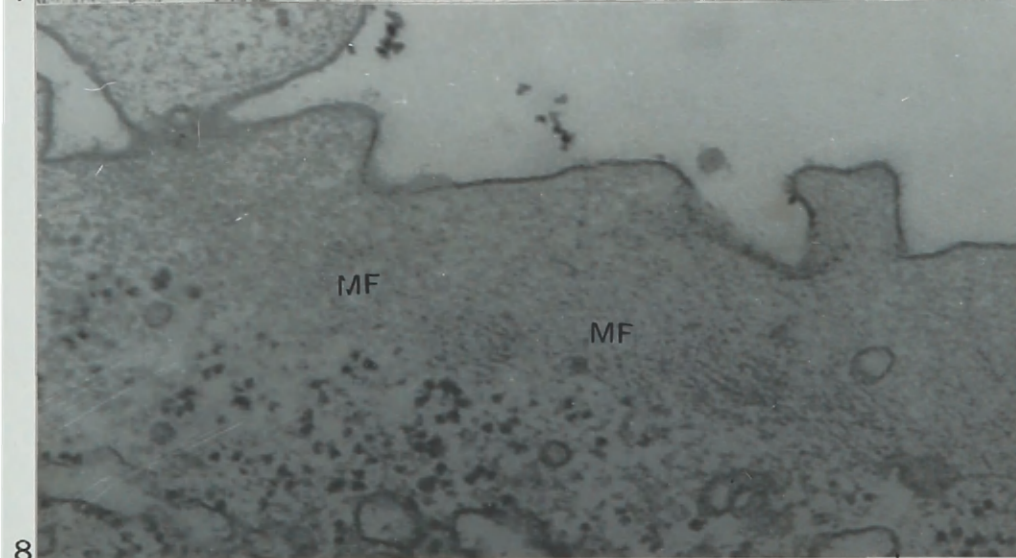
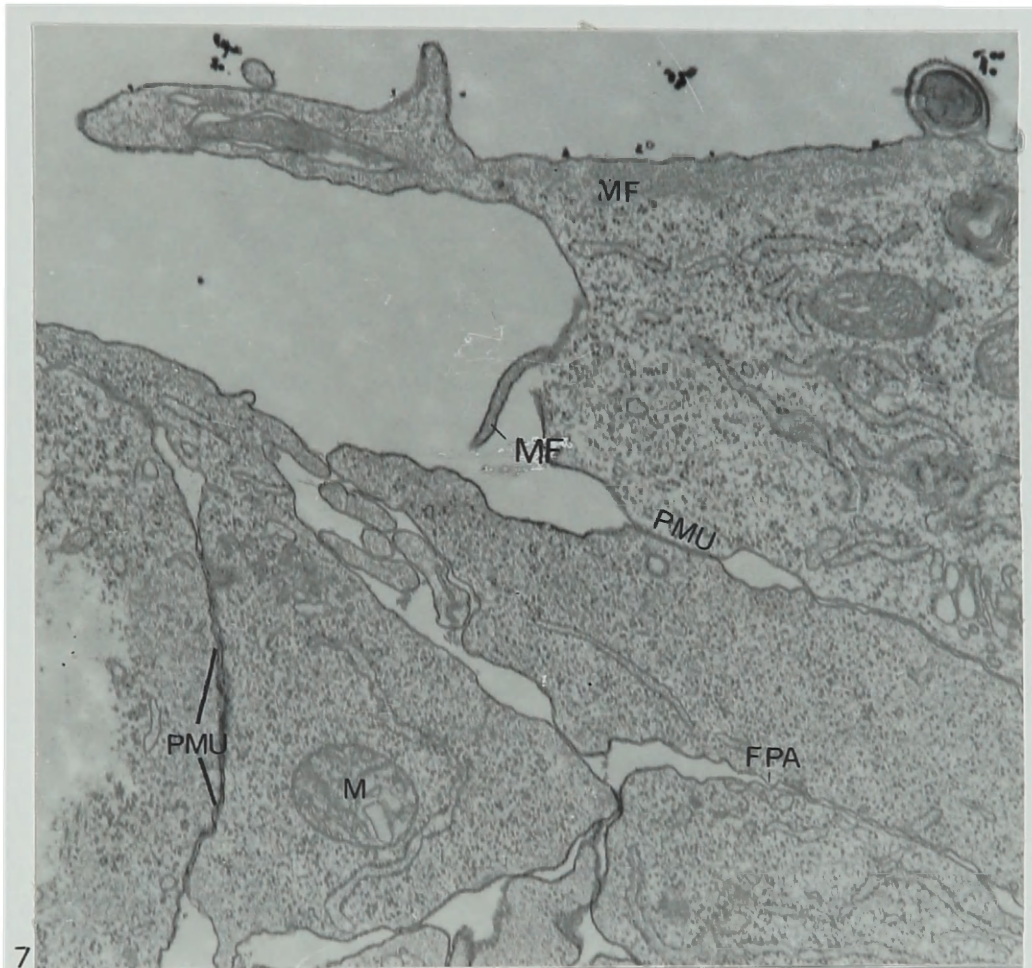


Figure 9 - Intact embryonic heart tissue mass showing normal ultrastructural relationships. (Mag. 3,300 X).

Figure 10- Control heart tissue mass showing normal ultrastructural relationships for reaggregates. (Mag. 6,300 X).

Figure 11- Enlargement of myofiber of heart reaggregate from control culture. (Mag. 28,000 X).

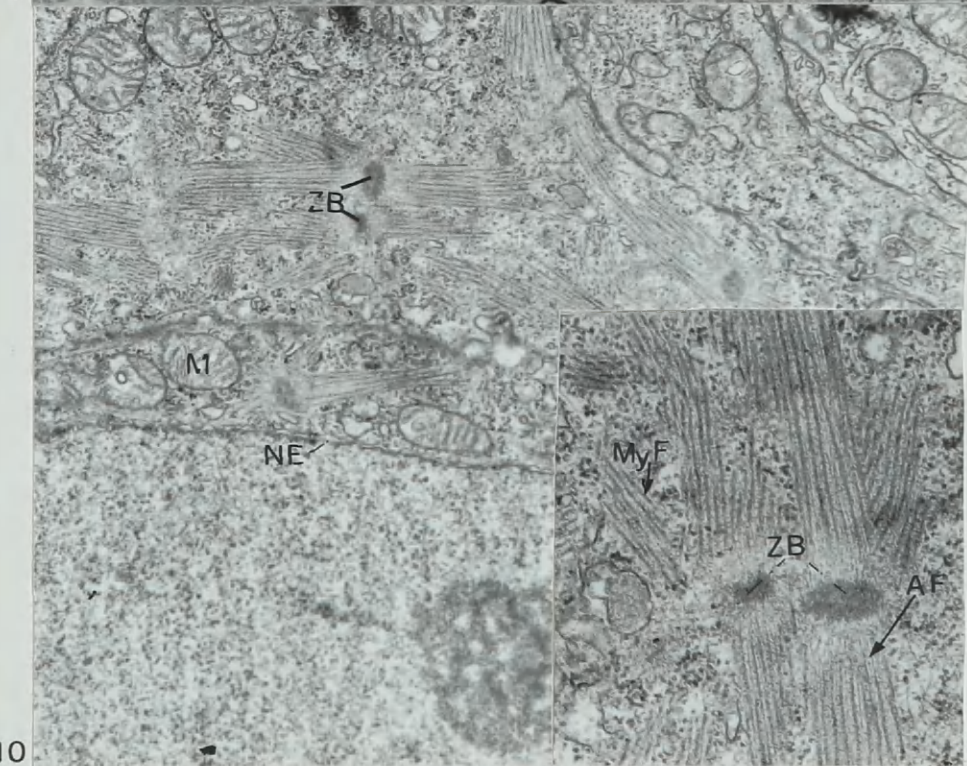
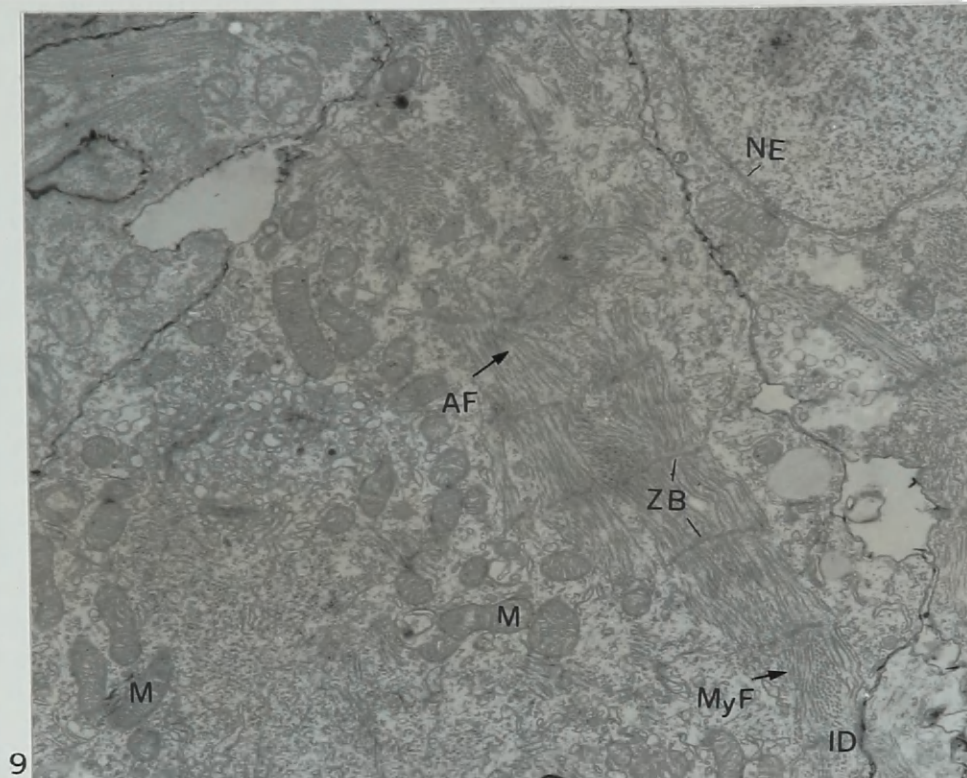
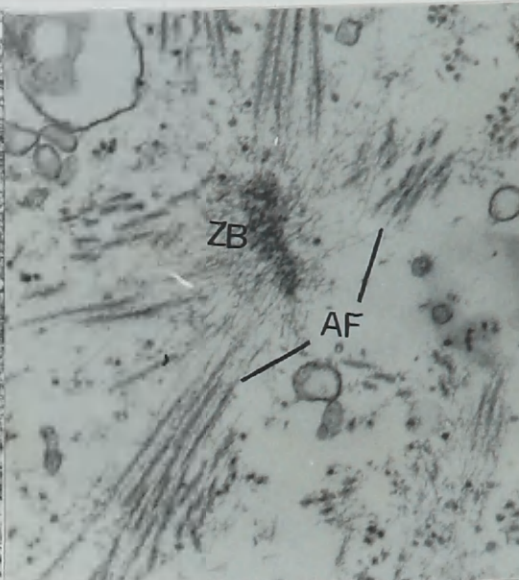
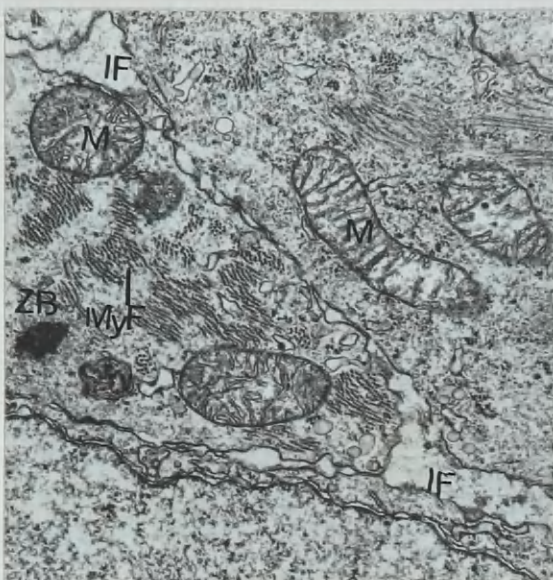


Figure 12 - 1 μ g heart tissue mass showing myofilaments.
(Mag. 16,600 X).

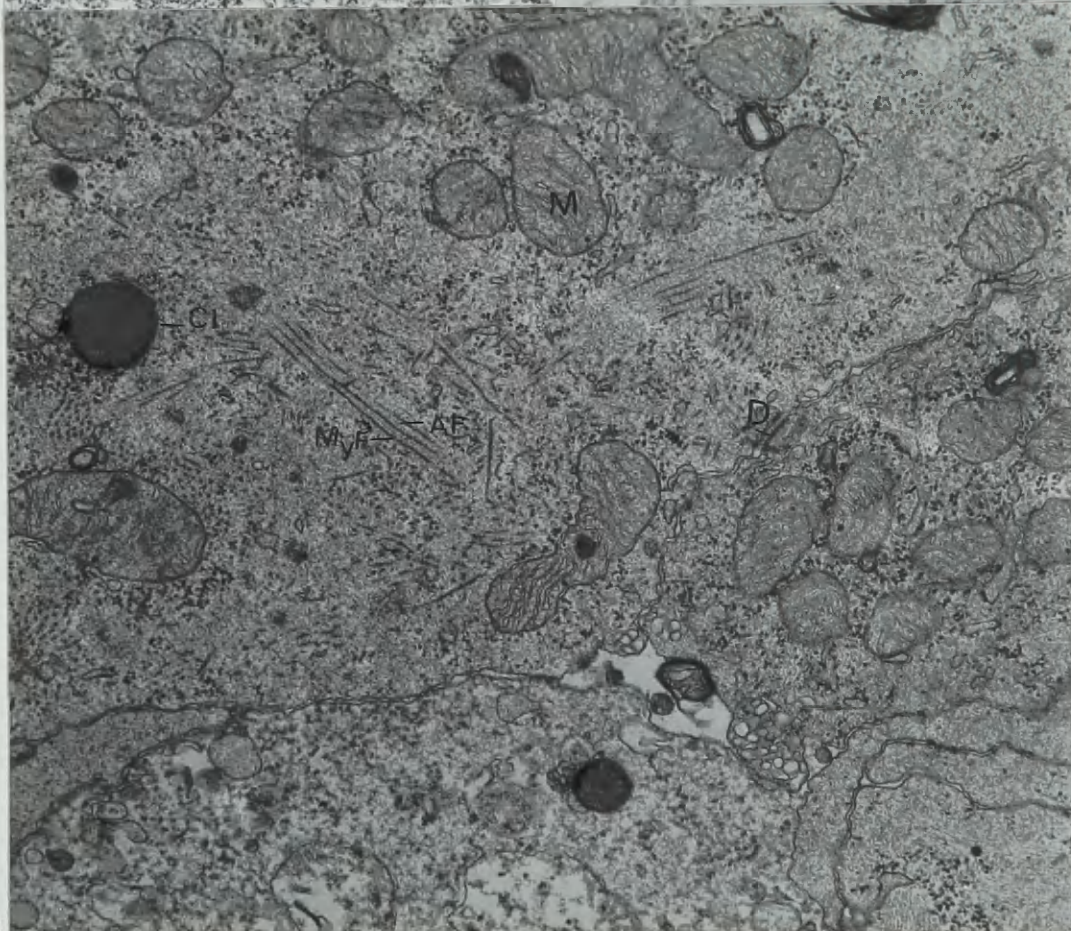
Figure 13 - 5 μ g heart tissue mass showing myofibrillar
disruption. (Mag. 49,000 X).

Figure 14 - 5 μ g heart tissue mass showing myofibrillar
orientation, dense intercellular fibers,
and cytoplasmic inclusions. (Mag. 19,000 X).

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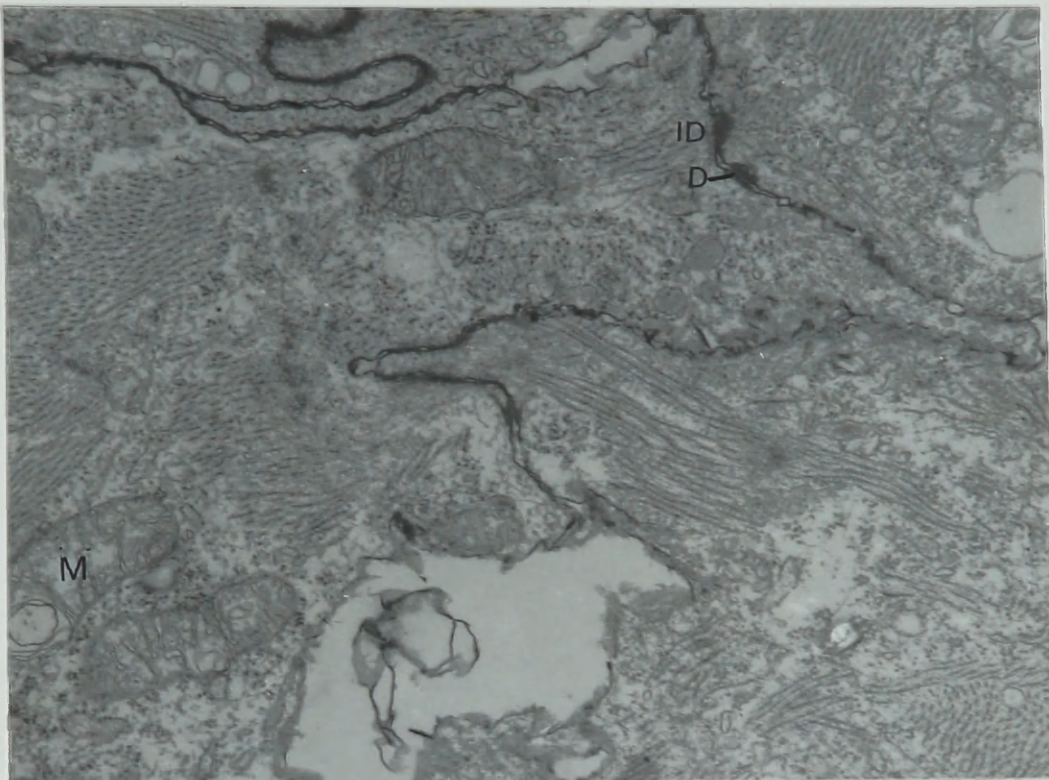


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Figure 15 - Intact heart reaggregate showing typical maculae adherens. (Mag. 15,900 X).

Figure 16 - 8 μ g heart reaggregate showing probable maculae adherens type junction. (Mag. 70,000 X).

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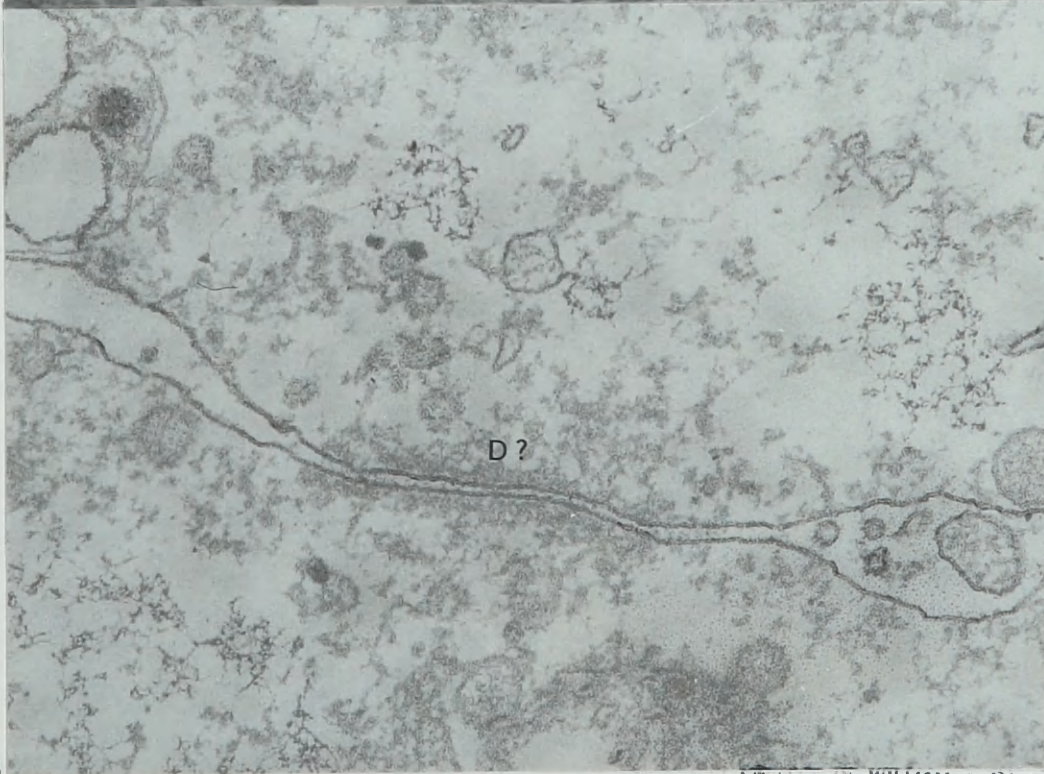
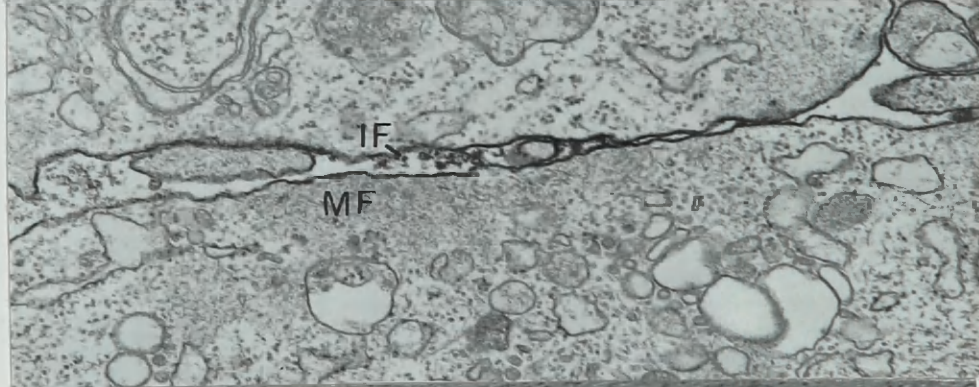
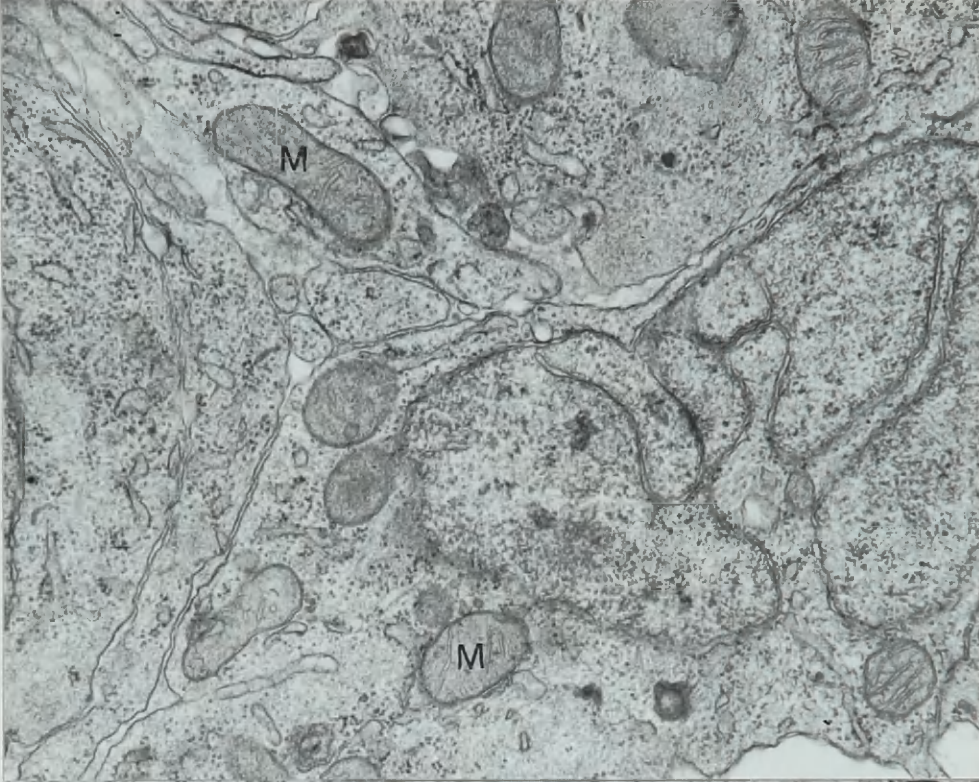


Figure 17 - 1 μ g liver recovery reaggregate showing dense intercellular fibers. (Mag. 28,500 X).

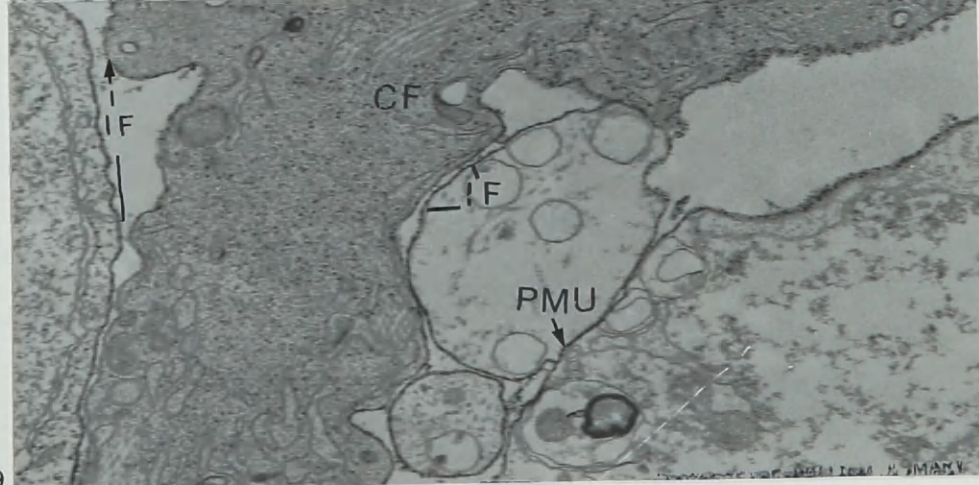
Figure 18 - 1 μ g liver tissue mass showing dense intercellular fibers, and parallel membrane units. (Mag. 28,500 X).

Figure 19 - 1 μ g heart tissue mass showing both intercellular microfibers and collagen fibers. (Mag. 19,000 X).

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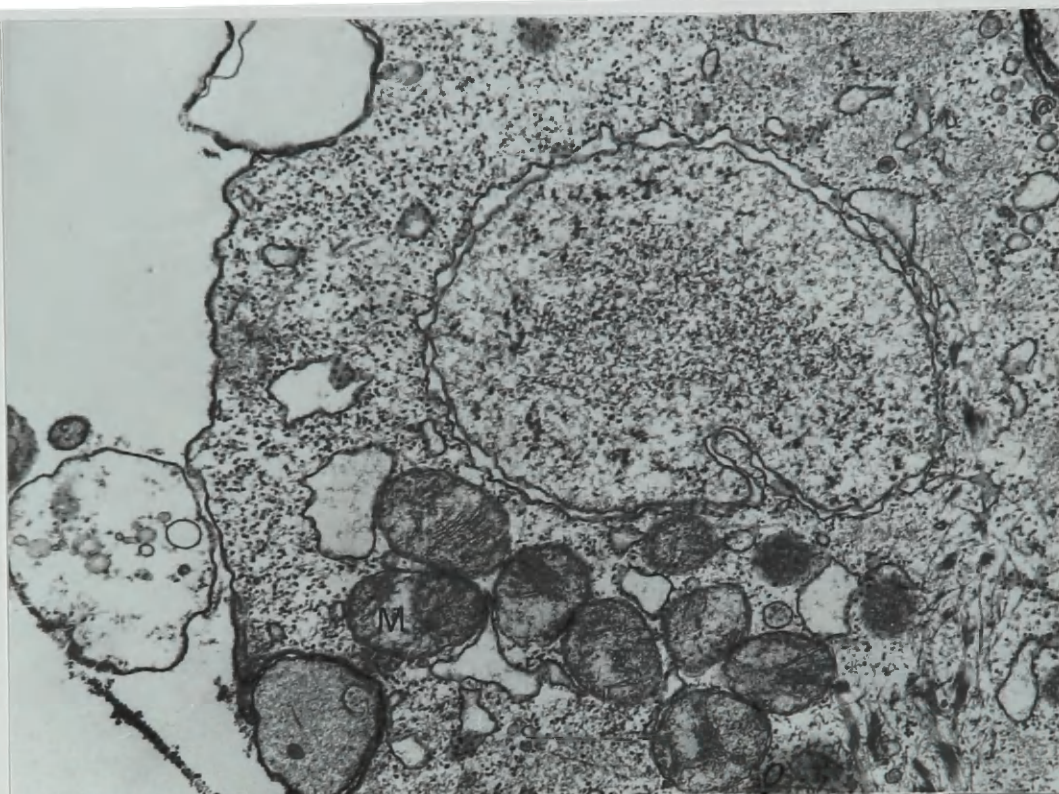


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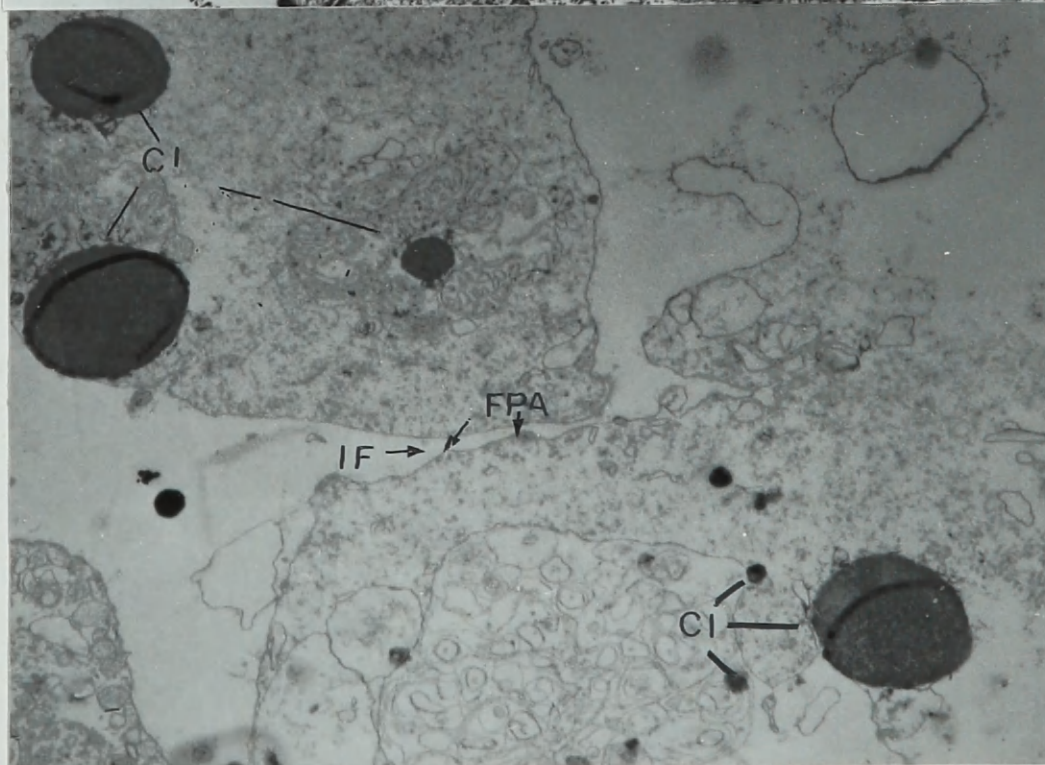
Figure 20 - 5 μ g liver tissue mass showing dense intercellular fibers and distended nuclear envelope. (Mag. 19,000 X).

Figure 21 - 8 μ g reaggregate showing the poor condition of cells, the cytoplasmic inclusions, and remnants of focal point adhesions. (Mag. 21,400 X).

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